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Improvement of Ergone Production from Mycelial Culture of *Polyporus umbellatus*

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Ergone, a fungal metabolite derived from ergosterol, was previously isolated and identified from *Polyporus umbellatus*. Ergone is a major component of *P. umbellatus* known to have anti-aldosteronic diuretic effect and also displays cytotoxic activities. Most of mushroom's fruit bodies used for test contained less than 10 μg/g of ergone. But *P. umbellatus* have larger amount of ergone than any other mushrooms. In order to improve the ergone production from the submerged culture of *P. umbellatus*, several factors including medium composition, culture conditions (temperature and pH) and different combinations of co-cultivation with various mycelia were studied. Among various carbon sources examined, starch proved to be most effective for the production of mycelia. The optimum pH and temperature for a flask culture of *P. umbellatus* mycelia were found to be 4.5 and 25°C, respectively. Under the optimized culture conditions, both the ergone production (86.9 μg/g) and mycelial growth (3.5 g/l) increased when *P. umbellatus* was cultured with *Armillariella mellea*. When the optimized conditions were applied, both mycelium and ergone production were significantly enhanced.

KEYWORDS: ***Armillariella mellea*, Co-culture, Ergone, Mycelial culture, *Polyporus umbellatus***

Ergone (ergosta-4,6,8(14),22-tetraen-3-one) is a metabolite derived from ergosterol, ergosterol peroxide and ergosta-6,22-diene-3β,5α,8α-triol by a set of multiple pathways (Price and Worth, 1974) (Fig. 1). It has been isolated previously from *Polyporus sclerotium, Dysidea herbacea, Lampteromyces japonicus* and some other Ganoderma species such as *G. lucidum* and *G. neo-japonicum* (Niedermeyer *et al*., 2005). It was also found in ergot sclerotia in barley and rye (Seitz and Pomeranz, 1983). Ergone has an anti-aldosteronic diuretic effect (Yuan *et al*., 2004) and displays cytotoxic activity against various tumor cell lines (Lee *et al*., 2005). Ergone was also studied in the screening of the marker compounds for the standardization of Polyporus Sclerotium (Yuan *et al*., 2003). For this reason, we have attempted to produce ergone from the *P. umbellatus* mycelium submerged culture.

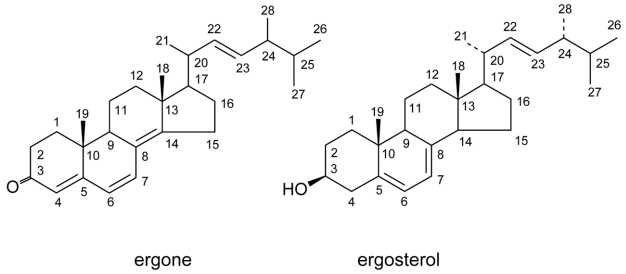

Fig. 1. Chemical structures of ergone and ergosterol.

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P. umbellatus (also called *Grifola umbellata*) is a mushroom, belongs to the Polyporales of the Basidiomycetes, and known causing a white rot in hardwoods. The sclerotium of *P. umbellatus* is a traditional Chinese medicine used for curing edema (Xiaoke and Shunxing, 2005). Whereas the water extracts of the sclerotia of *P. umbellatus* have been reported to have diuretic effects and its methanol extracts have cytotoxic effects against human gastric cancer cell. Various functional substances including erogostrol, erogosterol peroxide, ergone, glucan, and ergsta-7,22-dien-3-ol have been isolated from the sclerotia of *P. umbellatus* (Ohta *et al*., 1996).

Thus, it may be possible to produce those substances by cultivation of the mushroom. Submerged culture of fungi has a great potential to produce biomass and useful substances. Many fungi including *Boletus*, *Agaricus*, *Lentinus* have been successfully grown for the production of fungal mycelium. An advantage of adopting submerged culture over mushroom cultivation is its fast and controllable conditions (Belinky *et al*., 1994). Although other mycelia have been cultured for the production of mycelial biomass and useful substances such as lactic acid (Litchfield, 1997; Wee *et al*., 2005), citric acid (Papagianni *et al*., 1999) and methylanthranilate (Gross, 1990). *P. umbellatus* has not been tested for ergone production in culture. There are several reports where co-culturing of two microbes have been found to enhance enzyme or gallic acid production (Pandey *et al*., 1999; Purohi *et al*., 2006). However, no reports could find on the production of ergone by co-culturing of fungi. Many factors have been

implicated to affect on the production of the substance in culture. These include medium components, pH, and temperature. Here, we report optimum conditions and co-culture for the production of ergone from *P. umbellatus*.

Materials and Methods

Mycelia and inoculum preparation. The mycelia of *P. umbellatus* (KFRI 520), *Armillariella mellea* (KFRI 564), *Trihcoloma matsutake* (KFRI 438), *Ganoderma applanatum* (KFRI 646), *Lentinus edodes*, *Sparassis crispa*, and *Ramaria botrytis* used in this study were obtained from Korea Forest Research Institute. The stock cultures were maintained on potato dextrose agar slant. The seed cultures were grown in 250 *ml* flask containing 100 *ml* SYP medium at 25°C on a shaking incubator for 10 days. The cultured mycelia were homogenized at 13,000 rpm and used as inoculum.

Medium compositions and cultures. Mycelia were cultured in SYP medium containing the following composition (g/*l*): starch (16); fructose (4); peptone (1.5); formic acid (0.1) ;KH_2PO_4 (1); $MgSO_4$ (0.5); $FeSO_4$ (0.01); and yeast extract (1.5). Medium pH was adjusted to 4.5 before sterilization. One *l* flasks containing 400 *ml* of basal medium with 5% (v/v) of the mycelia of *P. umbellatus* were cultured on a rotary shaking incubator at 110 rpm. The cultivation was performed for 10 days at 25°C.

For the co-culture experiments, the first culture was grown in 1 *l* flasks containing 400 *ml* of basal medium with 5% (v/v) of the mycelia of *P. umbellatus* on a rotary shaking incubator at 110 rpm. After 3 days culture, other kind of mushroom mycelia (5% (v/v)) were added to the pre-cultured flask. The cultivation was performed for 15 days at 25°C.

Determination of ergone content. Sclerotium *of P. umbellatus* and fresh fruit bodies of *T. matsutake*, *G. applanatum*, *L. edodes*, *P. eryngii*, *S. crispa* and *R. botrytis* were lyophilized, and then powered to analyze for ergone. Cultured mycelia were separated from culture broth, washed by water, lyophilized, and then powered to analyze for ergone. The powered samples (3 g) were saponified by refluxing at 80°C for 30 min in 100 *ml* of methanol-ethanol (4 : 1, (v/v)) containing 10 g of KOH. The saponified solution was filtered and then 25 *ml* of distilled water were added to 60 *ml* of the filtrate. The mixture was partitioned with hexane. The hexane fraction was then evaporated to dryness, and dissolved with 3 *ml* of methanol. This sample was analyzed by HPLC (Thermo Separation Products) using 5 μm LiChrospher 100 RP-18 (26 × 8.0 mm) column. The UV spectra were recorded from 200 to 600 nm and the chromatograms were monitored at 300 nm by UV detector (TSP, spectrum system UV 3000HR). The elution consisted of 98% MeOH and 2% water and the flow rate was 1.2 *ml*/min. Ergone productivity (μg/*l*) was calculated as follows : Ergone productivity (μg/*l*) = Dry mycelial weight (g/*l*) × Ergone content (μg/g).

Table 1. Ergone content in mushrooms

Kind of mushroom	Parts	Content of ergone (μg/g)[a]
Polyporus umbellatus	Sclerotium	29.2 ± 0.64
Polyporus umbellatus	Mycelium	37.9 ± 2.64
Tricholoma matsutake	Fruit body	4.5 ± 0.16
Tricholoma matsutake	Mycelium	36.6 ± 4.39
Ganoderma applanatum	Fruit body	11.1 ± 1.57
Ganoderma applanatum	Mycelium	34.0 ± 2.64
Lentinus edodes	Fruit body	5.9 ± 0.59
Lentinus edodes	Mycelium	16.0 ± 1.29
Pleurotus eryngii	Fruit body	5.0 ± 0.58
Sparassis crispa	Mycelium	5.8 ± 0.15
Sparassis crispa	Fruit body	7.7 ± 0.85
Ramaria botrytis	Mycelium	25.8 ± 2.59

[a] All values are mean ± SD (n = 3).

Results and Discussion

Content of ergone in mushrooms. Ergone was known to be pharmaceutical substance for anti-aldosteronic diuretic (Yuan *et al.*, 2004). The component has been founded some kinds of mushroom (Niedermeyer *et al.*, 2005) and was also supposed to be contained any other mushrooms (Tanaka *et al.*, 1996). In order to compare ergone content of mushrooms and mycelia, we analyzed and determined ergone by HPLC (Table 1). Most of mushroom's fruit bodies used for test contained less than 10 μg/g of ergone except for *Ramaria botrytis*. Especially, parts of mycelium contained more amount of ergone than that of fruit body in a mushroom. However, *P. umbellatus* contained larger amount of ergone than other mushrooms tested (Table 1). From these results, we selected the mycelium of *P. umbellatus* for the production of ergone by submerged culture.

Effect of various carbon sources on mycelial growth and ergone production. Various carbon sources (glucose, starch, galactose, lactose, fructose, and sucrose) were compared for their effect on the production of mycelia (Fig. 2). Except for galactose and lactose, the addition of carbon sources significantly promoted mycelial growth. Among the various carbon sources examined, starch was most effective for the production of mycelia (3.8 g/*l*). However, neither galactose nor lactose supported the growth of *P. umbellatus* mycellium. Glucose and cellobiose were the most suitable carbon source in the submerged culture of *Ganoderma lucidum* (Yang and Liau,

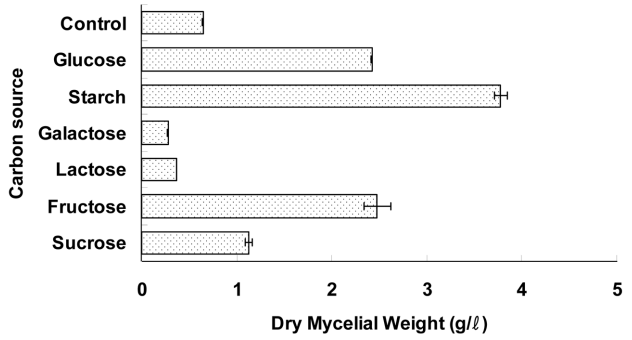

Fig. 2. Effect of carbon sources on the mycelial growth of *Polyporus umbellatus*. Cultivations were carried out in flasks for 10 days at 25°C. All values are mean ± SD (n = 3).

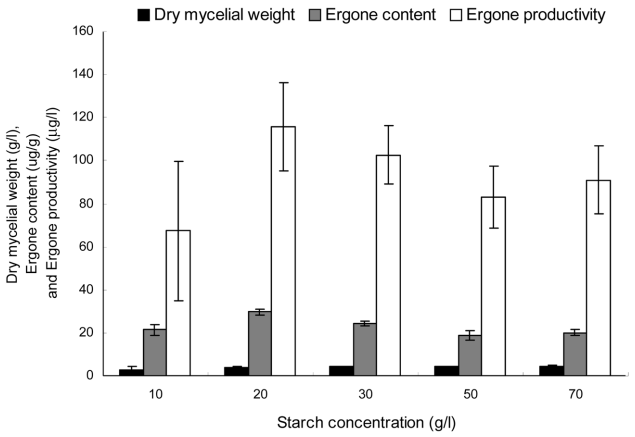

Fig. 3. Effect of starch concentration on the mycelial growth, ergone content and ergone production from *Polyporus umbellatus*. Cultivations were carried out in flasks for 10 days at 25°C. All values are mean ± SD (n = 3).

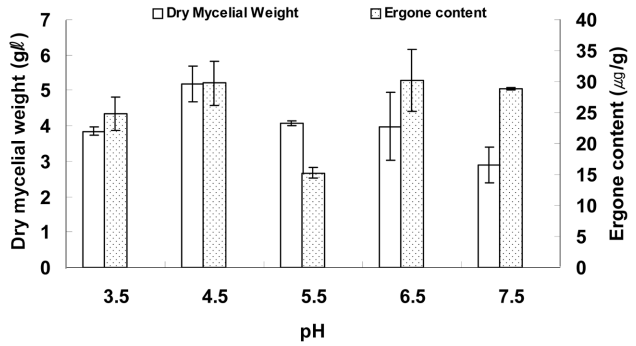

Fig. 4. Effect of pH for the production of mycelium and ergone from *Polyporus umbellatus*. Cultivations were carried out in flasks for 10 days at 25°C. All values are mean ± SD (n = 3).

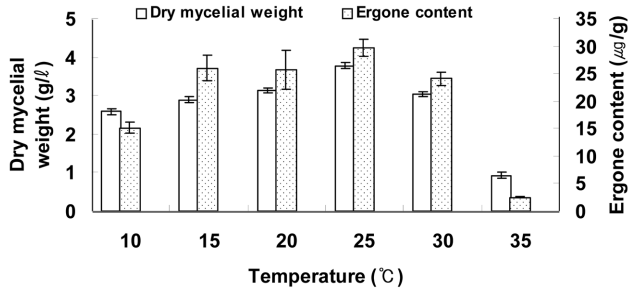

Fig. 5. Effect of temperature for the growth of mycelium and the production of ergone of *Polyporus umbellatus*. Cultivations were carried out in flasks for 10 days at different temperature. All values are mean ± SD (n = 3).

1998). However, in the present study starch proved to be the most favorable carbon source for the mycelial growth of *P. umbellatus*.

Based on the results as shown in Fig. 3, the mycelium of *P. umbellatus* was cultivated in the basal medium containing different amounts of starch to select the optimal level for the ergone production. The cultivation was performed for 10 days at 25°C. The dry mycellial weight of *P. umbellatus* increased with increasing starch concentration. The maximum mycelial growth was achieved in 70 g/*l* starch medium, while the maximum ergone content was obtained in 20 g/*l* starch medium (Fig. 3). Although, the mycelial growth was better in 70 g/*l* starch medium, the maximum ergone productivity (115.7 μg/*l*) was achieved in 20 g/*l* starch medium, since the ergone content of mycelia in 70 g/*l* starch medium (20.1 μg/g) was smaller than that in 20 g/*l* starch medium (29.8 μg/g). From the results as shown in Fig. 3, we propose that a suitable starch amount for the ergone production is 20 g/*l*. Whereas carbon sources affect cell growth, nitrogen sources influence cellular metabolism. Therefore, the optimization of both carbon and nitrogen sources is important in the production of mycelial biomass and their metabolite, ergone.

Effect of pH on mycelium growth and ergone production. The effect of initial pH of the media for the growth of mycelia and ergone production was determined. *P. umbellatus* was cultivated at different pH values in suspension culture for 10 days. As shown in Fig. 4, while mycelial growth was faster at pH 4.5 (5.2 g/*l*) than at any other pH value, the ergone content in the was larger at pH 6.5 (30.1 μg/g) than at any other pH values. It has been reported that many kinds of fungi of Polyporaceae family have more acidic pH optima during submerged culture and the mycelial growth of *P. umbellatus* was most favorable at pH 4 and most unfavorable at pH 9 (Shim *et al.*, 1997).

Effect of temperature on mycelium growth and ergone production. To determined the optimal temperature for ergone production, *P. umbellatus* was cultivated at various temperatures. Both the maximum mycelial growth

Table 2. Effect of pH for the production of ergone from *Polyporus umbellatus*

pH	Ergone productivity ($\mu g/l$)[a]
3.5	95.6
4.5	154.2
5.5	62.4
6.5	120.0
7.5	83.8

[a]Ergone productivity ($\mu g/l$) = Dry mycelial weight (g/*l*) × Ergone content ($\mu g/g$).

Table 3. Effect of temperature for the production of ergone from *Polyporus umbellatus*

Temperature (°C)	Ergone productivity ($\mu g/l$)[a]
10	39.3
15	75.2
20	80.1
25	112.5
30	73.0
35	2.3

[a]Ergone productivity ($\mu g/l$) = Dry mycelial weight (g/*l*) × Ergone content ($\mu g/g$).

(3.8 g/*l*) and the maximum ergone content (29.8 $\mu g/g$) were obtained at 25°C (Fig. 5). From the results, we were able to calculate the ergone productivity (Table 3). According to Shim *et al*. (1997), the mycelium of *P. umbellatus* favors 20°C for the growth and its related species also grow well at the temperature 24~27°C.

Co-culture with other mycelia for enhanced ergone production. Two sets of experiments were carried out to enhance ergone production : one with a pure culture of *P. umbellata*, *Armillariella mellea*, *Tricoloma matsutake* and *Ganoderma applanatum*, and the other with a co-culture of *P. umbellata* with other mycelia. For the co-culture experiments, the first culture was grown in basal medium with 5% (v/v) of the mycelia of *P. umbellatus*. After 3 days, other mycelia were added to the pre-cultured flasks. The cultivation was performed for 15 days at 25°C. This attempt to co-culture two kinds of mycelia was the first in the production of valuable metabolites. Table 4 shows the effect of co-culture with other mushroom mycelia for the ergone production. However, high ergone content in the mycelia did not lead to the higher production of the substance. For example, the ergone content of *Armillariella mellea* is the highest in the tested mycelia. However, total amount of ergone from the mycelia was less than that from *P. umbellatus*. This result is due to the difference in dry mycelium weight of the two mycelia. Therefore, as shown in Table 4, we conclude that both ergone production and mycelial growth increase when *P. umbellata* is cultured with *A. mellea*. The latter species is called as honey mushroom and known to be a pathogen to a wide range of woody plants (Tommerup and Broadbent, 1975). In contrast, both the production of ergone and mycelial growth decreased when *P. umbellatus* is cultured with *G. applanatum*. Further study is needed to elucidate the causes of the synergic effects of the co-culture.

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Table 4. Effect of co-culture with various mushroom mycelia for the production of ergone from *Polyporus umbellatus*[a]

Mycelia	Dry mycelial weight (g/*l*)[b]	Ergone ($\mu g/g$)	Ergone productivity ($\mu g/l$)[c]
Polyporus umbellatus	4.2 ± 0.29	29.8	125.2
Armillariella mellea	2.1 ± 0.16	43.3	90.9
Tricholoma matsutake	2.4 ± 0.29	28.5	68.4
Ganoderma applanatum	2.2 ± 0.42	24.3	53.5
P. umbellatus + *A. mellea*	3.5 ± 0.53	86.9	304.2
P. umbellatus + *T. matsutake*	3.6 ± 0.16	33.3	119.9
P. umbellatus + *G. applanatum*	2.3 ± 0.03	30.1	69.2

[a]Cultivations were carried out in flasks for 15 days at 25°C.
[b]All values are mean ± SD (n = 3).
[c]Ergone productivity ($\mu g/l$) = Dry mycelial weight (g/*l*) × Ergone content ($\mu g/g$).

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